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Method for Triggering Apoptosis in Cells

The present invention relates to a method of triggering apoptosis in cells, in particular in tumor cells.

Apoptosis is the programmed cell death. It is subject to accurate control, it being possible to induce or inhibit apoptosis.

As is known, apoptosis can be induced by a number of what is called death receptors, i.e. receptors containing a death domain (DD), such as CD95, TNF-RI, DR3, DR4 or DR5, which after binding their ligands induce apoptosis signal paths. For example, the CD95 receptor interacts with the adapter protein FADD/MORT1 after the binding of the CD95 ligand so as to induce the recruitment and the activation of the protease FLICE/Caspase-8 at the DISC "death inducing signalling complex". FADD and FLICE each contain death effector domains (DED). The induction of apoptosis via these apoptosis signal paths is possible from outside by e.g. the administration of cytotoxins (cytotoxic substances), irradiation, viruses, removal of growth factors or mechanical cell injuries. However, these possibilities of apoptosis induction are accompanied by certain drawbacks. For example, the administration of cytotoxins, such as cytostatic agents, or the irradiation in the case of cancer cells results in the development of a resistance and in addition in a damage of normal cells in which apoptosis should actually not be induced.

Hence it is the object of the present invention to provide a method serving for inducing apoptosis, e.g. to combat malignant growth, and simultaneously reduce the above described side-effects.

According to the invention this is achieved by the subject matters defined in the claims.

The present invention is based on the inventors' insights that in animals, in particular in mammals, more particularly in human beings, there is a protein which is suitable to induce apoptosis. Such a protein has a size of about 16 kD and has been characterized as a DNA-binding protein thus far (Nehls et al., Nucleic Acids Research, 26, pages 1160-1166 (1998).

The inventors recognized that the protein suitable to induce apoptosis (hereinafter referred to as C1D) is present in every cells, also in tumor cells, where it is expressed in an amount predetermined by the organism. Whenever the C1D gene product is overexpressed, apoptosis is induced in the overexpressing cells. However, especially in tumor cells apoptosis obtained by overexpression is desired. This overexpression *per se* can kill the tumor cells. Furthermore, it could enhance the apoptosis effected by common tumor treatment, such as a chemotherapy or irradiation. Moreover, apoptosis could be effected in tumor cells in which a resistance has already developed by common therapeutic methods. The inventors now found that apoptosis can be induced by overexpressing the C1D gene, i.e. increasing the concentration of the cellular C1D gene product. This can be done e.g. by transfecting the cells with expression constructs which express the C1D gene or by stimulating overexpression of the endogenous C1D gene.

The C1D gene product comprises the sequence of fig. 1 or 2 or an amino acid sequence differing therefrom by one or several amino acids. The expression "an amino acid sequence differing therefrom by one or several amino acids" comprises any amino acid sequence coding for a C1D (related) protein, whose DNA sequence hybridizes with the DNA of fig. 1 or fig. 2. As to the expression "hybridizes" reference is made to the below explanations.

A nucleic acid coding for C1D in the form of a DNA, in particular a cDNA, is particularly suitable to carry out the method according to the invention. A DNA is preferred which comprises:

(a) the DNA of fig. 1 or 2 or a DNA differing therefrom by one or several base pairs, the latter DNA hybridizing with the DNA of fig. 1 or 2, or

(b) a DNA related to the DNA from (a) via the degenerated genetic code.

The sequence data of the C1D cDNAs according to fig. 1 and 2 are available in the gene library under the following accession numbers:

Mouse cDNA: X95591;
Human cDNA: X95592.

The expression "a DNA differing by one or several base pairs" comprises any DNA sequence coding for a C1D (related) protein, which hybridizes with the DNA of fig. 1 or 2. The DNA may differ from the DNA of fig. 1 or 2 by additions, deletions, substitutions and/or inversions of one or several

base pairs or other modifications known in the art, e.g. alternative splicing. According to the invention the expression "DNA" also comprises fragments of this DNA. The expression "fragment" shall comprise a segment of the original nucleic acid molecule, the protein encoded by this fragment still comprising the apoptosis-inducing properties of C1D. This also comprises allele variants. A person skilled in the art is familiar with methods of producing the above modifications in the nucleic acid sequence, and such methods are described in standard works of molecular biology, e.g. in Sambrook et al., Molecular Cloning: A laboratory manual, 2nd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY (1989).

The person skilled in the art can also determine whether a protein encoded by a nucleic acid sequence modified in this way still has the biological activity of inducing apoptosis, e.g. by the detection of apoptosis-typical cell death characterized by e.g. morphology, multicentric chromatin condensation, typical membrane changes and endogenous DNA degradation.

The expression "DNA hybridized with ..." refers to a DNA which hybridizes with a DNA of fig. 1 or 2 under common conditions, in particular 20°C below the melting point of the DNA. In this connection, the expression "hybridizes" refers to conventional hybridizing conditions, preferably to hybridizing conditions under which 5xSSPE, 1 % SDS, 1x Denhardt's solution is used as a solution and the hybridization temperatures are between 35°C and 70°C, preferably at 65°C. Following the hybridization washing is carried out first with 2xSSC, 1 % SDS and then with 0.2xSSC at temperatures between 35°C and 70°C, preferably at 65°C (for the definition of SSPE, SSC and Denhardt's solution see

Sambrook et al., *supra*). Stringent hybridization conditions, as described e.g. in Sambrook et al., *supra*, are particularly preferred.

In order to produce the C1D gene product which is suitable for carrying out the method according to the invention, the DNA coding for C1D is inserted in an vector or expression vector, e.g. pBlueScript, pQE8, pUC or pBr322 derivatives. In a preferred embodiment, the nucleic acid molecule according to the invention is functionally linked in the vector with regulatory elements which permit its expression in eukaryotic host cells. Such vectors contain, in addition to the regulatory elements, e.g. a promoter, typically a replication origin and specific genes which permit the phenotypic selection of a transformed host cell. The regulatory elements for the expression in eukaryotes include the CMV, SV40, RVS40 promoter, and the CMV or SV40 enhancer. Further examples of suitable promoters are the metallothionein I and the polyhedrin promoter.

In an embodiment preferred for gene-therapeutic purposes, the vector including the C1D DNA is a virus, e.g. an adenovirus, vaccinia virus or adeno-dependent parvovirus (AAV). Retroviruses are particularly preferred. Examples of suitable retroviruses are MoMuLV, HaMuSV, MuMTV, RSV or GaLV. For the purposes of gene therapy the nucleic acid molecules according to the invention can also be transported to the target cells in the form of colloidal dispersions. They comprise e.g. liposomes or lipoplexes (Mannino et al., Biotechniques 6 (1988), 682).

General methods known in the art can be used for the construction of expression vectors, and in particular gene therapy vectors, which contain the above-mentioned nucleic

acid molecules and suitable control sequences. These methods comprise e.g. *in vitro* recombination techniques, synthetic methods and *in vivo* recombination methods as described e.g. in Sambrook et al., *supra*. Thus, the person skilled in the art knows how to insert a DNA according to the invention in an expression vector. He is also familiar with the fact that this DNA can be inserted in combination with a DNA coding for another protein or peptide, so that the DNA according to the invention can be expressed in the form of a fusion protein, e.g. in the form of a fusion protein in which the other part is GFP (the green fluorescent protein of *Aequorea Victoria)*.

For the expression of the C1D gene the above-mentioned expression vectors are introduced into host cells. The host cells comprise animal cells, preferably mammalian cells, in both culture and the living organism. The animal cells L, 3T3, FM3A, CHO, COS, Vero and HeLa are preferred. Methods of transforming these host cells, detecting the transformation as taken place, and expressing the nucleic acid molecules according to the invention by using the above-described vectors are known in the art.

In addition, the person skilled in the art knows conditions under which transformed and transfected cells can be cultured. He is also familiar with methods of isolating and purifying the protein or fusion protein expressed by the DNA according to the invention.

In order to carry out the method according to the invention the C1D DNA is introduced in a preferred embodiment in an expression vector, in particular a gene therapy vector, and in cells, preferably tumor cells. This is where the expression of the C1D protein occurs, which in addition to

the cell-inherent protein results in the induction of apoptosis. The vectors are introduced into the cells under conditions with which the person skilled in the art is familiar. As to the *in vivo* gene therapy reference is made in particular to "K.W. Culver, Gene Therapy, A Handbook for Physicians, Mary Ann Libert, Inc., New York, 1994" and "P.L. Chang, Sonatic Gene Therapy, CRC Press, London, 1995".

In another preferred embodiment the cell-inherent C1D gene is stimulated to an increased expression, e.g. by exogenous stimulation of the endogenous C1D promoter. 5'-neighboring sequences of a gene are referred to as a promoter, which serve as starting points of RNA polymerase II which in cooperation with the transcription factors effect the expression of the gene. In many genes, and also in C1D, this process can be induced or stimulated by exogenous factors. Factors which effect the specific expression of a gene are very numerous and range from physical factors (such as light, heat, cold) via low-molecular inorganic substances (such as salts, metal ions) and low-molecular organic substances (peptides, nucleic acid building blocks, biogenic amines, steroids) to polymolecular substances (serum, growth factors, immunostimulators). The stimulators specific of the C1D gene are recognized by combining 5'-neighboring sequences, present on e.g. the BAC (bacterial artificial chromosome) clones, with a reporter gene, e.g. CAT or EGFP, and examining them for the reporter gene expression and/or its stimulation by exogenous factors, optionally by means of a high-throughput method.

Thus, the present application provides for the first time the possibility of triggering apoptosis not via the common signal paths but through overexpression of a certain gene. This can be of special significance for many diseases, in

particular for tumoral diseases. The fact that tumor cells respond to an overexpression of C1D with much more sensitivity than normal cells proved to be especially advantageous. Therefore, no side-effects exist for normal cells, whereas tumor cells undergo safe cell death.

Brief description of the figures:

Fig. 1 shows the DNA and amino acid sequence of C1D from a human,

Fig. 2 shows the DNA and amino acid sequence of C1D from a mouse,

fig. 3 shows the temporal course of an apoptosis process triggered by overexpression of C1D in cells of the Ehrlich ascites tumor (fluorescence microscopy; excitation: 480 nm; emission: 520 nm)

fig. 4 shows examples of morphological characteristics occurring in the course of an apoptosis process triggered by overexpression of C1D in cells of the Ehrlich ascites tumor (phase contrast pictures).

The present invention is explained by the below examples.

Example 1: Induction of apoptosis by expression of the C1D gene

- pcDNA 3 - C1D expression constructs

The cDNA cloned in the Bluescript vector (KS+, company of Stratagene) and coding for human or murine C1D was amplified by PCR. In this connection, the following primers were used:

For human cDNA:

Primer forward:
5'-GGGGTACCATGGCAGGTGAAGAAATTAATGAAGACTAT

primer reverse:
5'-GGGTCGACTTAACTTTTACTTTTTCCTTTATTGGCAAC

(effects the amplification of the nucleotide sequence from base 118 to base 540 according to figure 1)

For mouse cDNA:

Primer forward:
5'-GGGGTACCATGGCAGGTGAAGAAATGAATGAAGATTAT

primer reverse:
5'GGGTCGACGTGTTTGCTTTTCCCTTTATTAGCCACTTT

(effects the amplification of the nucleotide sequence from base 78 to base 500 according to figure 2)

By means of these primers a Kpn restriction site was introduced before the ATG start codon and a Sal I restriction site was introduced before the stop codon (so that the stop codon was omitted). The PCR reaction was carried out by means of the PCR kit from the Clontech company (K1906-1) according to the manufacturer's instructions using the kit components in 50 µl volumes:

Water	38.8 µl
10x buffer	5 µl
Mg acetate	2.2 µl
Primer forward	1 µl (1 µM)
Primer reverse	1 µl (10 µM)
C1D template	5 µl (500 ng)

50x dNTP	1 µl
kit polymerase	1 µl

cycler program:

1)	initial denaturation	94°C, 1 min
2)	denaturation	94°C, 30 sec
3)	annealing extension	68°C, 3 min
4)	final extension	68°C, 3 min
5)	cooling	4°C

steps 2)/3) are carried out 35 times.

Following the restriction digestion of the amplification batch using Kpn I/Sal I, the fragments were initially recloned in the Bluescript vector (Kpn I/Sal I - pretreated). After excising the fragments from the Bluescript vector using Kpn I/Not I, the sequences could be cloned into the correspondingly pretreated pcDNA 3 vector (InVitrogen company).

- pcDNA 3-C1D-EGFP expression constructs

The fusion between C1D and GFP (green fluorescent protein of *Aequorea Victoria)* with continuous reading frame was effected on the pBluescript level. For this purpose, the above described pBluescript-(Kpn I)-C1D-(Sal I)-plasmids were opened by digestion with Sal I/Hind III.

The sequence coding for EGFP (company of Clontech; EGFP means "enhanced green fluorescent protein" and is a mutant produced by the Clontech company, which has properties improved as regards the excitation/emission) was amplified by PCR. In this PCR, the following primers were used:

Primer forward:

5'-GGGTCGACATGGTGAGCAAGGGCGAGGAGCTGTTC

primer reverse:
5'-CCAAGCTTTGGAATTCTAGAGTCGCGGCCGCTTTA

to insert a Sal I site at the 5-end and a Hind III site at the 3'-end (here after the stop codon). The PCR was carried out analogously as described above.

After the digestion of the PCR amplification products using Sal I and Hind III they could be ligated into the prepared Bluescript-(Kpn I)-C1D-(Sal I) ... (Hind III) plasmids. Thereafter, the fusion cassette (Kpn I)-C1D-EGFP-(NotI) was excised by corresponding digestion out of the Bluescript vector and recloned into the correspondingly pretreated pcDNA 3 vector (Kpn I/Not I).

- Transfection of the vectors in tumor cells

The above-obtained expression vectors were separated from one another by means of electroporation (Potter et al., Proc. Natl. Acad. Sci. USA, 81, pages 7161-7165 (1984) or lipofection (SuperFect Transfection Reagent Handbook, Quiagen company, Hilden, 02/97, 1997) in cells of the Ehrlich ascites tumor. Transfected (living cells) were observed in the microscope (fluorescence optical system) and photographed (fig. 3).

About 12 hours after the transfection, 20 - 60 % have a weak green fluorescence in the cell nucleus (not shown). This refers to the initially moderate expression of the fusion protein. From a morphological point of view no characteristics can be seen. Approximately 24 hours after the transfection of the vector construct agglomerations of the fusion protein occur in individual cells (fig. 3 left

side). These agglomerations can also be observed in the phase contrast picture (fig. 4). In the further temporal course, these agglomerations become stronger (fig. 3, from left to right) and the phase contrast picture (fig. 4) corresponds to the typical picture of a cell undergoing apoptosis.

Not all of the cells which were simultaneously transfected show the excess expression rate of the fusion protein in the picture at the same time. Cells such as on the left side in fig. 3 are also observed even after 48 - 72 hours, whereas other cells have already reached their end point (fig. 3, right side). This shows that the cells of a culture enter the apoptosis process "in staggered manner". With a sufficiently high transfection rate all cells of a culture, i.e. also those which were not transfected, are killed in the final analysis. This instant depends on the initial transfection rate. By the apoptosis of the transfected cells factors are released which are harmful for non-transfected cells in the culture and finally result in the killing of these non-transfected cells as well (what is called "bystander effect").

It is to be remarked that GFP (green fluorescent protein of *Aequorea Victoria)* was only used to make a distinction between transfected and non-transfected cells or to make visible the overexpression. GFP expression alone has no effect on the cell morphology or the ability of survival of cells. GFP fusion proteins have basically the functional properties (and also the intracellular distributions) equal to the functional gene product. The apoptotic processes shown in the figures are therefore based on a C1D function. The morphology shown (and the loss of cell number) was effected in control experiments also by constructs which

only included the C1D sequence (e.g. by the above described pcDNA 3-C1D expression constructs).